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Are hypothalamic neurons transsynaptically connected to porcine adipose tissue?

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Abstract

Specific anatomical sites and pathways responsible for mediating metabolic and neuroendocrine effects of leptin are still poorly understood. Therefore, we examined distribution of leptin receptor-containing neurons transsynaptically connected with the porcine fat tissue by means of combined viral transneuronal tracing and immunohistochemical staining method. Pseudorabies virus (PRV) was injected into the perirenal fat tissue in pigs, and after survival periods of 3, 5, 7, 9, and 11 days, hypothalami were processed immunohistochemically with primary antisera against PRV and leptin receptor (OBR). PRV labeled neurons were found in paraventricular nucleus (PVN), ventromedial nucleus (VMN), anterior hypothalamic area (AHA), preoptic area (PA), arcuate nucleus (ARC), and supraoptic nucleus (SON) by nine days after injection of the virus. Double-labeling immunofluorescence demonstrated that OBR were co-localized in nearly all virus-infected neurons. The present results provide the first morphological data demonstrating a multisynaptic circuit of neurons of CNS origin which innervates porcine fat tissue.
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Keywords: Hypothalamus; Leptin receptor; Transsynaptic neural labeling; Fat tissue; Pig

The mechanism for food intake regulation at the level of the central nervous system (CNS) is poorly understood in large animals. The discovery of the ob gene and anti-obesity effect of leptin was a breakthrough in understanding the role of adipose tissue in regulation of food intake, body weight, and endocrine function [1]. It is currently hypothesized that leptin is secreted from adipose tissue, circulates in the blood, and acts at the brain to reduce food intake, increase energy expenditure, and alter endocrine activity [2–4]. Leptin provides a link between adipose tissue and the brain. The hypothalamus is the primary target of leptin action, since the neuroanatomical substrate of central leptin actions mainly coincides with several hypothalamic cell groups, which include at least four nuclei in the medial hypothalamus; the paraventricular (PVN), arcuate (ARC), ventromedial (VMN), and dorsomedial (DMN) nuclei [5,6]. In particular, NPY producing neurons, which are present in ARC, express leptin receptors and administration of leptin results in down-regulation of NPY gene expression, reduced feed intake, and increased energy expenditure [7]. Moreover, leptin may also affect other types of neurons, such as those modulating the sympathetic nervous system [5,6,8–10].

Recent neuromorphological studies using a viral transsynaptic tracing method [11] revealed that nerve cells within the CNS are transsynaptically connected to different organs including fat tissue [12–22]. This information provides the basis to study the functional significance of CNS structures in the control of adipose tissue metabolism. Reports regarding the above-mentioned method in domestic animals are lacking, especially with respect to adipose tissue.

Therefore, the purpose of the present study was to establish whether or not hypothalamic leptin receptor-containing neurons are transsynaptically connected to porcine perirenal adipose tissue.

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Materials and methods

The principles of animal care published by the National Institutes of Health (publication No. 86-23, revised 1985) as well as the governmental regulations of Poland (Ustawa o ochronie zwierzat Dz. U. nr 111 z 21. 08. 1997) were followed. Eighteen female pigs of the Large White Polish breed weighing 50 ± 2 kg were pre-treated with atropine (Polfa, Poland; 0.04 mg/kg b.w., s.c.) and propionyl–promasine (Combelen, Bayer, Germany; 0.4 mg/kg b.w., i.m.). Thirty minutes later, sodium pentobarbital (Vetbutal, Biovet, Poland; 30 mg/kg b.w.) was given intravenously to induce surgical anesthesia. The Pseudorabies virus (PRV, Bartha's K strain, 3.0×10^9 plaque forming units per milliliter) was introduced in ten injections of 2 μl each evenly distributed on the surface of the right perirenal fat tissue depot in 15 of the pigs using a Hamilton syringe by way of the paralumbar fossa. Special care was taken to avoid entry of virus into surrounding tissues. The volume of injected virus was based on preliminary studies. In the control group ($n = 3$), perirenal fat tissue was injected with 0.1 M phosphate-buffered saline (PBS; pH 7.4). After a survival period of 3, 5, 7, 9, and 11 days, animals ($n = 3$ pigs per group) were anesthetized as described above and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Control pigs were sacrificed 11 days after injections. Brains were collected and hypothalami were dissected out after making the following cuts: rostral to the optic chiasm, rostral to the mammillary bodies, lateral to the hypothalamic sulci, and ventral to the anterior commissure. Tissue blocks were then postfixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 2–3 h at 4 °C and transferred into 30% sucrose and 0.01% NaN_3 until they sank. Ten micrometer thick frozen sections were mounted on slides, processed by double-labeling immunofluorescence [23] with primary antisera against PRV (Mouse # P01510, dilution 1:800, Institut Pourquier, France) and OBR (Goat # sc-1833, dilution 1:400, Santa Cruz Biotechnology, USA), and photographed with a Leitz Orthoplan or a Zeiss Axiophot fluorescence microscope equipped with epiillumination and an appropriate filter set for Texas Red and FITC. The specificity of primary antisera was tested with preabsorption control sections. In brief, 1 μM of the respective peptide completely abolished fluorescence and there was no immunostaining observed in the absence of primary antisera.

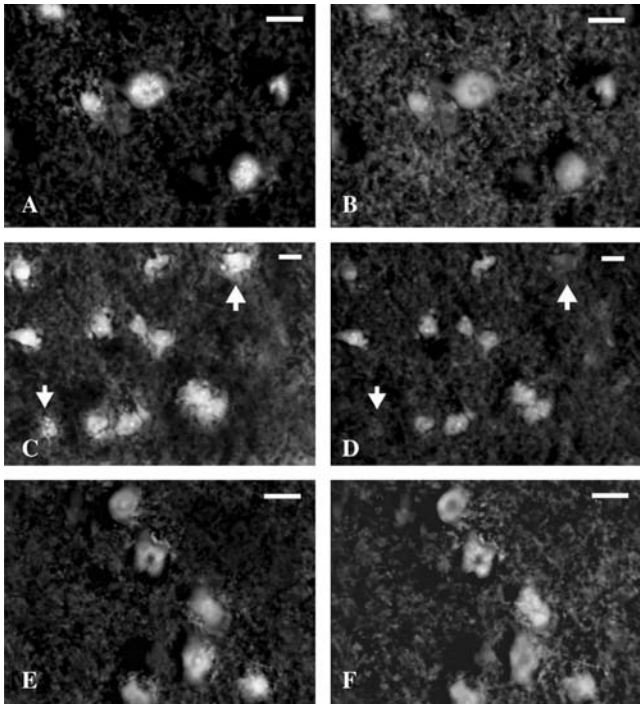

Fig. 1. (A,B) Group of PRV-IR neurons located in PVN (A) simultaneously exhibiting OBR-IR (B). Scale bar = 20 μm. (C,D) Numerous PRV-IR neurons located in ARC (C) exhibiting OBR-immunoreactivity (D). Two of PRV-infected cells located in the ARC not exhibiting OBR-immunoreactivity (arrows). Scale bar = 20 μm. (E,F) PRV-IR neurons located in VMN (E) simultaneously exhibiting OBR-IR (F). Scale bar = 20 μm.

Results and discussion

In the present study, PRV-infected neurons were located in the PVN, anterior hypothalamic area (AHA), preoptic area (PA), supraoptic nucleus (SON), ARC, and VMN by 9 days after injection of the virus. In the PVN, PRV-IR neurons were moderate in number (5–10 labeled cells per section). Neurons located in the AHA-PA (Fig. 1A) represented the smallest subpopulations of labeled cells (1–5 cells per section). The most abundant PRV-IR cell bodies were located in the ARC and SON (10–30 cells per section, Fig. 1C). A moderate number of PRV-IR neurons were found in the VMN (5–10 labeled cells per section; Fig. 1E). There were no PRV-immunoreactive neurons in the hypothalami collected from control pigs. These results support the idea of a transsynaptic connection between hypothalamic neurons and perirenal adipose depot. The pattern of infected neurons after PRV injections was similar to that found in the hamster and rat [12,14,24,25]. However, the number of infected neurons and the period of virus migration differed between studies. The difference in the number of infected neurons may have resulted from different titers of PRV used as well as mass of the fat tissue depots [12–14,24,25]. The main advantage of neural tracing with PRV compared to monosynaptic tracers [8,26] is that this method allows a temporal analysis of the viral progression through synaptically linked neurons in the CNS after injection into a peripheral organ. In the present study, PRV-infected neurons were found in the hypothalamus by 9 days after injection of the virus, while in laboratory animals this period varied between 5 and 6 days [12,14]. Therefore, the speed of virus migration may depend on both the extent and dimension of nerve fibers supplying the organ under study.

Another useful feature of PRV transsynaptic retrograde tracer is that the phenotype of PRV-infected neurons can be characterized by double-labeling immunohistochemistry. Double-labeling immunofluorescence demonstrated that OBR were co-localized in almost all virus-infected hypothalamic neurons. OBR immunoreactivity was observed in PVN, AHA, PA (Fig. 1B), SON, ARC (Fig. 1D), and VMN (Fig. 1F) of the porcine hypothalamus. No immunoreactive structures were found in the median eminence. All OBR-IR neurons were PRV-infected (Figs. 1A–F) while some PRV-infected cells located in the ARC (1–4 neurons per section) did not

exhibit OBR-IR (Figs. 1C and D). The presence of OBR-IR neurons in the porcine hypothalamus confirms a previous report in which semiquantitative reverse transcription-polymerase chain reaction was used to identify OBR gene expression in the hypothalamus of gilts [27]. In addition, our results demonstrate the presence of OBR-IR neurons in the PA and AHA, which are in agreement with previous reports in the sheep, rat, mouse, and pig [5,28–30]. The present study demonstrated that the greatest number of OBR-IR cell bodies was located in the SON and ARC. In contrast to the present results and others obtained from pigs [5], OBR-IR neurons in the rat SON [28] were not strongly immunoreactive. The ARC is proposed as a major site of action for leptin based on the present results as well as those of many previous studies [5,27–30]. Moreover, the present results are in agreement with previous reports in domestic animals demonstrating that OBR is present in neurons of both the PVN and VMN. Thus, these nuclei may regulate outflow of information and may be the autonomic and endocrine effectors of leptin action [5,29]. Immunohistochemical and physiological studies revealed that projections of OBR-IR neurons provide a link between the hypothalamus and other brain regions involved in satiety and reproductive functions [2,5,27,31,32]. These findings suggest that leptin not only plays an integrative role in feeding behavior, but also in neuroendocrine activity. It is clear, however, that the definitive assignment of physiological roles for leptin requires further pharmacological as well as molecular investigations. Nevertheless, pharmacological intervention directed at the OBR may prove to be a novel and effective treatment for diseases such as anorexia nervosa and obesity. Additional research is needed to develop a complete understanding of the adipose tissue–brain–pituitary axis, which will lead to practical methods of controlling appetite and metabolism in farm animals as well as in humans.

The present results provide the first morphological data on the multisynaptic circuit of neurons innervating porcine fat tissue and demonstrate that hypothalamic leptin receptor-containing neurons are transsynaptically connected to the perirenal fat depot.

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